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EFFECT OF THE BIOFLAVONOID SILYMARIN ON THE IN VITRO ACTIVITY AND EXPRESSION OF SUPEROXIDE DISMUTASE (SOD) ENZYME

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Superoxide dismutase activity and expression of the erythrocytes and lymphocytes of patients suffering from chronic alcoholic liver disease and those of healthy controls were investigated after in vitro incubation with silymarin. It was concluded that silymarin treatment in a concentration achievable by in vivo treatment (10 μg/ml) significantly increased the SOD activity of both the erythrocytes and lymphocytes of patients with liver disease, whereas the SOD expression of the lymphocytes enhanced to a considerable extent.

These results indirectly indicate that the scavenger silymarin is able to increase the antioxidant protection of the cells by ameliorating the deleterious effects of free radical reactions.

Keywords: silymarin, superoxide dismutase, antioxidant, hepatoprotection, alcoholic liver disease

Reactive free radicals play an important role in the development of toxic liver damages of various origin [1, 3, 11, 17]. Every aerob cell is equipped with complex defense mechanisms for the elimination of the potentially toxic oxygen intermediers [4]; one of these mechanisms is the superoxide dismutase enzyme (SOD, E.C.1.15.1.1.) [8, 12]. In our previous studies it has been demonstrated that in patients suffering from chronic alcoholic liver disease, SOD activity of the blood cells is decreased and SOD expression of the lymphocytes is also diminished [15, 21]. Our observations are in accordance with the results of other research groups who reported on decreased SOD activity of the erythrocytes, a datum similar to that seen in investigations on liver tissue samples [30, 37, 38].

The natural flavonoid silymarin (Legalon®, Madaus, Germany) is a free radical scavenger with antioxidant characteristics [33, 34], and has been found to be an effective substance against certain forms of experimental liver injuries [6, 33, 34]. The present project was aimed at studying the in vitro effect of silymarin on antioxidant protection both in patients with chronic alcoholic liver disease and in healthy controls.

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Patients and methods

Twelve patients (10 males and 2 females, mean age: 37 ± 9 yr) suffering from chronic alcoholic liver disease were involved in the study. Daily average alcohol consumption exceeded 60 g in males and 30 g in females, whereas the average duration of chronic alcoholism was over 4 years. Liver biopsy was carried out in every patient and showed micronodular cirrhosis in 9 cases, while fatty liver together with fibrosis in 3 cases. Fifteen healthy blood donors (11 males and 4 females, mean age: 39 ± 6 yr) were studied as controls.

Among the components of the antioxidant defense system superoxide dismutase activity of the blood cells and SOD expression of the lymphocytes were determined before and after in vitro silymarin (Legalon®) treatment.

Silymarin was used in a concentration of 10 μg/ml, which — according to Hirschaut's formula [13] — corresponded to the usual therapeutic dosage achievable by the traditional in vivo administration of the drug. Silymarin dissolved in PBS was present in the test systems during the whole duration of the incubation (18 hours).

SOD activity was measured by the original method of Misra and Fridovich [20], i.e. by inhibiting the spontaneous autotransformation of adrenalin. The colour reaction of the adrenochrome formed was detected spectrophotometrically at 480 nm wave length. Erythrocytes were obtained by centrifugation of 10 ml of venous blood anticoagulated with heparin. The erythrocytes sedimented were washed three times with 5 ml of isotonic saline solution. Red cell lysates were prepared by adding 0.9 ml of distilled water to 0.1 ml of packed erythrocytes. Hemoglobin was precipitated with 0.8 ml of chloroform-ethanol (3 : 5 v/v) mixture. The pale-yellow supernatant obtained after centrifugation was used for estimating the specific activity of SOD [18, 32].

Lymphocytes were separated on Ficoll gradient. Phagocytes were removed by carbonyl-iron treatment. Viability of the cells exceeded 98% when measured by trypan blue staining. Monocyte contamination was less than 2%. For enzyme determination lymphocyte membranes were disrupted in ice-cool PBS by using sonication. After centrifugation SOD analysis was performed from the supernatant. All data were expressed in units of total SOD/ml sample.

For the flow-cytofluorimetric evaluation of SOD expression on lymphocyte membranes cells were labelled with monoclonal mouse anti-calf Cu/Zn SOD antibody [22]. The antibodies bound were detected by using a second antibody conjugated with FITC (Dako, Denmark). The distribution-intensity histograms were recorded by means of an FC 4800 A cytofluorograph (Bio Physics System, Inc. USA).

SOD expression on lymphocyte membranes can be defined as the degree of epitope presence recognized by monoclonal anti-SOD antibody. The linkage has been found to be specific since native (unlabelled) SOD prevented it in a dose-dependent fashion however inhibition of Fc receptors failed to influence it. Moreover, Cu/Zn SOD was observed on human liver cell membrane by immunoperoxidase technique, as well [27].

For statistical analysis Student's paired and unpaired *t* tests were used.

Results

SOD activity was found to be considerable lower in the blood cells of patients suffering from chronic alcoholic liver disease than in those of the controls (Fig. 1). The cytofluorimetrically detected SOD expression of the lymphocytes proved to be affected, too (Fig. 2).

Silymarin in an in vitro concentration of 10 μg/ml increased significantly the SOD activity of both the erythrocytes and lymphocytes of patients with chronic alcoholic liver disease ($p < 0.01$ for erythrocytes and $p < 0.05$ for lymphocytes, respectively). Moreover, SOD activity of the erythrocytes practically returned to the normal level (Fig. 1). Following incubation for 18 hours no change of considerable extent could be detected as far as the SOD activity of the blood cells originating from healthy subjects was concerned (Fig. 1).

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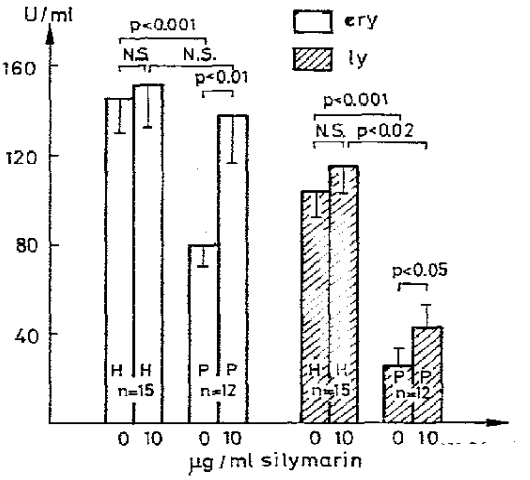

Fig. 1. Effect of in vitro silymarin treatment on the SOD activity of erythrocytes and lymphocytes from patients with chronic alcoholic liver disease (mean unit/ml ± SEM, ery: erythrocyte, ly: lymphocyte, H: healthy, P: patient)

In vitro silymarin treatment practically normalized the pathologically diminished SOD expression of the lymphocytes in patients with chronic liver disease (Fig. 3). However SOD expression of the lymphocytes in case of healthy controls exhibited only a slight increase (Fig. 4).

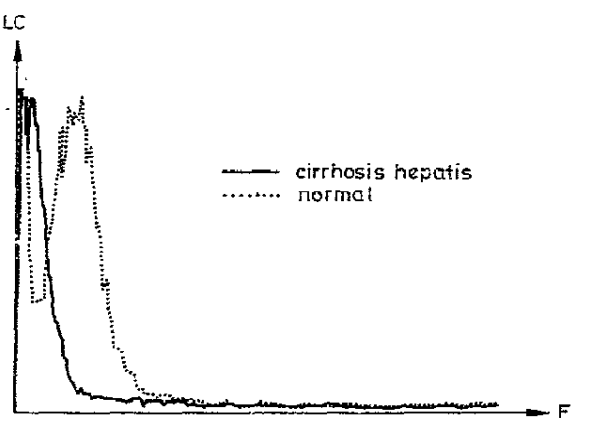

Fig. 2. SOD expression of lymphocytes from healthy subjects and patients with chronic alcoholic liver disease measured by flow-cytofluorimetry. Fluorescence histogram (LC: lymphocyte count, F: fluorescence intensity)

Discussion

Toxicity of free radicals produced during alcohol metabolism plays a substantial role in the pathogenesis of alcoholic liver disease [10, 11, 26, 35]. Reactive oxygen intermediers inducing lipid peroxidation may finally cause

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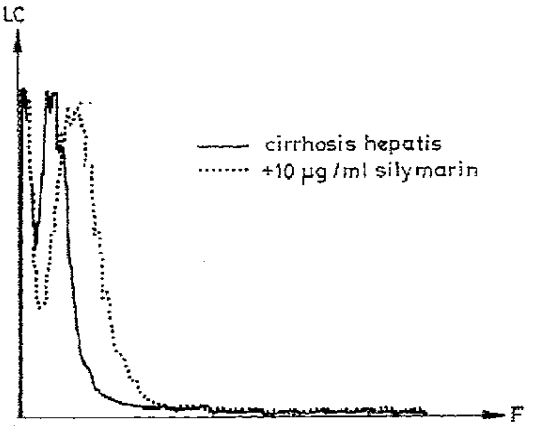

Fig. 3. Effect of in vitro silymarin treatment on the SOD expression of lymphocytes from patients with chronic alcoholic liver disease measured by flow-cytofluorimetry. Fluorescence histogram (LC: lymphocyte count, F: fluoresecence intensity)

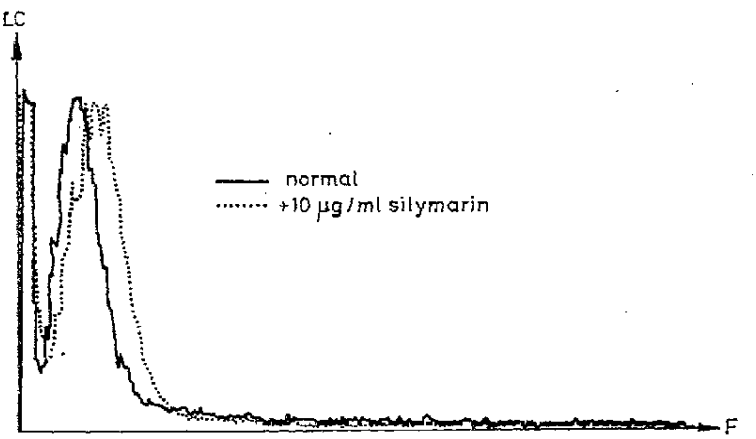

Fig. 4. Effect of in vitro silymarin treatment on the SOD expression of lymphocytes from healthy controls measured by flow-cytofluorimetry. Fluorescence histogram (LC: lymphocyte count, F: fluorescence intensity)

the destruction of the cells [9]. The organism possesses specific protective systems against oxidative damages [4], nevertheless, even these mechanisms may be impaired in chronic liver diseases [15, 19, 21, 25, 28]. In chronic alcoholic liver injury oxygen stress and the consequent lipid peroxidation are limited not solely to the damaged liver cells but, due to the effect of the relatively stable, diffusable and poisonous end-products of lipid peroxidation (e.g. malondialdehyde, 4-hydroxynonenal), manifest themselves also extrahepatically [2, 7, 24, 31]. Or to put it an other way, the process may also involve such cells and tissues which, at first, were not directly exposed to peroxidative damages.

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Since highly reactive oxygen species and the increased lipid peroxidation probably play a causative role in alcoholic liver disease it should be possible to mitigate this damage with agents that scavenge free radicals and exert antioxidant and membrane stabilizing effects. Recently the naturally occurring bioflavonoid cyanidanol-3 (Catergen®) has been widely used in its treatment [23] but the severe toxic side-effects observed, however, limited the further use of the drug in human therapy.

Nowadays the therapeutic administration of another structurally similar flavonoid, namely silymarin is spreading in the hepatologic practice. Similarly to (+)-cyanidanol-3, its basic action is the well-documented free radical scavenger, antioxidant and membrane protective activity [33, 34]. In this study we investigated the in vitro effects of silymarin on the superoxide dismutase enzyme activity and expression of isolated peripheral blood cells from both patients suffering from chronic alcoholic liver injury and healthy controls. SOD has been considered to be the central component of intracellular enzyme systems capable of eliminating toxic free radicals [8, 12].

The pathologically decreased basal SOD activity of the erythrocytes and lymphocytes as well as the SOD expression of the lymphocytes in patients with alcoholic liver disease enhanced significantly after incubation with silymarin.

Generally in alcoholic liver disease the concentration of SOD enzyme protein corresponds to enzyme activity [5]. On the basis of the present study, close correlation can be supposed between the SOD activity and expression of the lymphocytes. The effects of silymarin demonstrated in this investigation may indirectly suggest the free radical scavenger and antioxidant capacity of the drug [32, 33]. On the other hand, as far as SOD activity and expression of the lymphocytes is concerned, the influence of the compound to increase protein biosynthesis can not be excluded either [29].

The originally decreased SOD activity and expression in patients with alcoholic liver disease, among others, may be due to excess of free radicals produced during alcohol metabolism (i.e. by partial inhibition of the enzyme activity by substrate overproduction) leading to limited adaptive capacity. On the other hand, the diminished SOD activity and expression may also reflect the enzyme inhibitory (inactivating) effect of the overproduction of hydrogen peroxide, which is actually the end-product of the enzymic reaction catalized by SOD [14]. In addition, a lipid peroxidation-dependent depression of de novo protein synthesis could also be a pathogenic factor since protein synthesis is considerably affected by chronic alcohol consumption [16, 36].

Since in healthy organisms the reactive oxygen species produced are under the control of physiological protective mechanisms, it is understandable that the enzyme activity and expression of intact cells showed hardly any alterations at all after incubation with silymarin.

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Summing up, it can be concluded that as an effect of in vitro silymarin treatment, the antioxidant capacity of the blood cells originating from alcoholic patients suffering from liver disease increased considerably. Therefore this compound seems to be a promising possibility in the therapeutical problems of alcoholic liver injury unsolved so fare. Detailed clinical studies are needed, however, for the final conclusion to be drawn.

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